ARTICLE ADDENDUM

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Probiotic manipulation of the gastrointestinal microbiota

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In a recent publication we examined whether high abundance of a probiotic species, *Lactobacillus casei* subsp. rhamnosus GG (LGG), impacted the overall composition of the gastrointestinal (GI) microbiota of six-month-old infants at high risk for asthma development. Profound GI microbiota restructuring and the establishment of significantly more even and putatively, functionally redundant consortia were characteristic of high LGG abundance. Here we discuss, in the context of more recently published data, support for the hypothesis that the beneficial effect of probiotic supplementation on human health lies in the formation of a stable and resilient gut ecosystem enriched for species that exert a concerted beneficial effect on the host immune system via direct and indirect mechanisms.

The presence and composition of the complex microbial community in the gastrointestinal (GI) tract has long been implied to play a crucial role in human physiology and health.[1-3] In addition to the local effects the gastrointestinal microbiome exerts on gut physiology and immune response,[4-6] this assemblage has more recently been shown to influence systemic immune responses and host physiology at extra-intestinal sites.[6-8] The presence and abundance of specific species within the microbial consortium has been associated with the development of chronic inflammatory diseases, e.g., inflammatory bowel disease (IBD), and also with the risk for, and development of, allergy and asthma which manifest at sites remote from the gut.[9,10] Thus an improved understanding of the role of the GI microbiota in development and maintenance of immune homeostasis is critical for development of novel strategies to combat both intestinal and extra-intestinal chronic inflammatory diseases.

There is growing evidence that environmental microbial exposures, appropriate gastrointestinal microbial colonization, and host sampling of the developing GI microbiota in early infancy are crucial to immune response maturation and allergic disease outcomes.[11,12] A growing number of studies have demonstrated that early events in microbial GI tract colonization during infancy precede the development of allergies and asthma later in life.[13-19] Thus, the emerging paradigm, based largely on the hygiene hypothesis, is that early stimulation of the immature immune system by a diversity of commensal microbes during the crucial stage of immune maturation is required for the development of a balanced immune system.[20,21] Therefore, a lack of exposure to microbial antigens or inappropriate colonization due to host genotype or external selective pressures (e.g., antibiotic use) increases the likelihood of allergic disease development. That this process depends on microbial exposures is further supported by our recent findings that dog ownership, which has previously been associated with protection against allergic disease development, is associated with a significant increase in house dust bacterial diversity and lower fungal richness, compared to homes with no pets.[22] Moreover, although the numbers were small in this study, the microbial composition of the house dust could largely be differentiated on the basis of pet behavior. House dust from homes with pets (dogs or cats) who were permitted both in- and outdoors were associated with a diverse array of bacterial species of which the most frequently detected belonged to the main phyla found in the gastrointestinal tract, raising the possibility that these exposures may serve as an inoculum for the developing gastrointestinal microbiota. In comparison, dust from houses with animals who stayed exclusively in- or out-doors, or had no pets, exhibited significantly reduced bacterial diversity coupled with the presence of more fungal ribotypes, many of which are known allergenic species. Hence, growing evidence supports the prevailing hypothesis that early infant microbial exposures influence immune response maturation and subsequent development of allergic disease.

Approaches to modulate microbial exposure and manipulate the composition of the gastrointestinal microbiota during the crucial period of immune development to promote appropriate maturation, has largely been based on supplementation with known beneficial bacterial species. Given the newly recognized importance of gastrointestinal colonization and microbiota composition at this site, it is fortunate that there exists a long history of research efforts on human supplementation with probiotics, i.e., non-pathogenic microbial species that exert host beneficial effects. However, it is only in recent years that sufficiently sophisticated culture-independent tools have been developed to examine the impact of such interventions on the complex human gastrointestinal microbiota, an emerging key component in development and maintenance of immune homeostasis. Several reports have demonstrated that infant supplementation with probiotics results in decreased risk of atopic disease development.[23-26] Exposure during infancy to *Lactobacillus casei* subsp. rhamnosus GG (LGG), a native of the gastrointestinal microbiota and frequently used as a probiotic, resulted in decreased rates of atopic eczema in probiotic-supplemented children compared to control subjects who received placebo.[15] In animal models, *Lactobacillus reuteri* supplementation has been shown to attenuate the respiratory inflammatory response and to reduce allergen-induced skin inflammation in sensitized mice,[27,28] demonstrating a clear role for gastrointestinal microbial manipulation in abrogating inflammatory conditions at sites remote from the gastrointestinal tract. However, the mechanisms by which these species exert their effect and whether efficacy depends solely on the supplemented species or on a GI microbial consortium effect, remain largely unknown.

To begin to understand the fundamental processes of how probiotic supplementation may impact allergic disease development, we recently analyzed the fecal microbiota of six-month-old infants participating in the Trial of Infant Probiotic Supplementation (TIPS) study using a high-resolution, culture-independent phylogenetic microarray.[29] TIPS is a randomized, placebo-controlled, double-blind study aimed to assess the effect of daily probiotic supplementation with LGG on the development of early markers of atopy.[30] This study, led by Dr. Michael Cabana MD at the UC San Francisco, in which stool samples are collected under standardized conditions from an extremely well characterized cohort of neonates, sampled periodically from birth through the first year of life, and whose allergic status is determined at 6 years of age, provides an ideal opportunity to examine the mechanism(s) by which microbial supplementation impacts the gastrointestinal microbiota in human subjects and impacts allergic disease development outcomes.

Babies enrolled in the TIPS study received daily supplements of LGG or placebo from birth to six months. Stool samples collected at 6 months from a subset of neonates in the study were examined using the 16S rRNA PhyloChip, a high density microarray capable of identifying approximately 8,500 bacterial taxa in a single, standardized assay.[31-33] Using the array we were able to detect a much greater diversity of bacteria in the infant stool than previously reported using a clone library approach,[34] a phenomenon we and others have demonstrated in several other studies.[32,35] Members of 46 bacterial phyla were detected, primarily representing the Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes which is in agreement with previous reports of human gastrointestinal community composition.[36-38] Bacterial community richness (i.e., number of taxa present in a single sample) was similar across all samples and averaged at about 1,146 taxa ± 125. Importantly, these data indicated that a high abundance of LGG did not result in domination of the community by this species to the detriment of other bacterial community members. However, exploratory statistical analysis demonstrated that those samples with a high abundance of LGG exhibited a distinct microbial community structure from those with low abundance of this organism, demonstrating for the first time that probiotic abundance, and presumably associated activity, acts as a selective pressure on microbiota composition. Comparative analysis of samples with the highest abundance of LGG versus samples with the lowest abundance demonstrated significant differences in the abundance of 682 taxa—all were more abundant in the high-abundance LGG samples.

Strikingly, the majority of the taxa promoted in high LGG samples were phylogenetically clustered and relatively closely related. Recently, a study performed in mice has demonstrated this phenomenon of "like begets like" in which communities with keystone species either beneficial or infectious are more likely to be successfully colonized by closely related species.[39] In an attempt to identify the characteristics of a gut microbiota protective against gastrointestinal infection, Stecher and colleagues tested the colonization resistance of mice with various gut microbial assemblages, to oral inoculation with virulent *Salmonella enterica* serovar Typhimurium. Murine microbiota susceptible to Salmonella colonization and infection were characterized by a higher abundance of Enterobacteriaceae. Follow-up experiments demonstrated that successful Salmonella colonization of these microbiota was positively correlated with *Escherichia coli* abundance. This led the authors to propose that the chance of newly incoming species to enter and colonize a well-established gut ecosystem is increased when closely related bacteria are already abundant in that niche. The study went on to show that this principle also applied to commensal bacteria; mice with high abundances of Lactobacilli were more efficiently colonized by the closely related commensal *Lactobacillus reuteri* upon oral inoculation. Indeed we have also demonstrated this phenomenon in the airways of older cystic fibrosis (CF) patients who typically possess microbial communities comprised of multiple members of the Pseudomonadaceae that exhibit significantly greater phylogenetic relatedness in their microbiota compared to younger CF patients.[40] This suggests that while distinct microbial assemblages may inhabit discrete human host niches, their invasion resistance is, at least in part, predicated on the composition of the native microbiota colonizing this niche, a phenomenon that has enormous implications for microbial manipulation strategies for chronic disease management as well as protection against acute infection, e.g., Methicillin-resistant *Staphylococcus aureus* (MRSA) carriage and infection potential.

To further expand this hypothesis and identify community members that exhibited co-varying abundance with LGG, correlation analysis was performed, revealing 358 positive and only 3 negative correlations. Among the promoted taxa were known probiotic species such as *Lactobacillus fuchuensis* and *Bifidobacterium bifidum*. However, many of the positive correlations identified were with poorly characterized species, suggesting that there is a large untapped reservoir of species present in the gastrointestinal tract that require systemic examination and characterization to define their role as potential immunomodulatory organisms. Our study demonstrated that contrary to pathogen strategies which include domination of the microbial community,[41] supplementation with probiotic species promotes the abundance of a large diversity of bacterial species and increases community evenness, which putatively increases functional redundancy and resilience of the ecosystem. More specifically, data from the TIPS study suggests that the positive effect of LGG on human health might not (only) be due to the high abundance of the supplemented species but to the global changes in the bacterial community structure that this inoculum elicits. Consequently, the beneficial effects of probiotics may be the result of concerted activity on immune responses by several bacterial species whose abundance is promoted due to the presence or activity of the supplemented probiotic species.

Support for the hypothesis that the efficacy of probiotic supplementation is due to the activity of multiple species, comes from a report recently published in the *Proceedings of the National Academy of Science*.[42] In an attempt to investigate how probiotics affect the host immune response, Kwon and colleagues tested several candidate probiotic strains (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Bifidobacterium bifidum* and *Streptococcus thermophilus*) for their anti-inflammatory potential in a murine model. More specifically, they were interested in identifying the strain that elicited the most robust increase in the population of $CD4^+$ regulatory T cells (Tregs) expressing the transcription factor Foxp3, necessary for the development and function of Tregs, in murine mesenteric lymph nodes. $CD4^+Foxp3^+$ Tregs suppress activation of the immune system thereby maintaining immune homeostasis, and as such are the central control point in the regulation of autoimmune responses.[43] Interestingly, Kwon and colleagues found that supplementation with a mixture of the five probiotic strains (called IRT5) as opposed to administration of single species conferred the most potent anti-inflammatory activity both in vitro and in vivo. Moreover, administration of IRT5 to mice suppressed the progression of intestinal and extra-intestinal autoimmune disorders, inflammatory bowel disease, atopic dermatitis and rheumatoid arthritis. Strikingly, four of the five species used in the IRT5 mix were amongst the taxa most significantly enriched in the gastrointestinal microbiota of infants who possessed a high abundance of LGG in our TIPS study, supporting the hypothesis that the concerted activity of multiple species is necessary to elicit an immunomodulatory effect. Further support comes also from clinical trials investigating the efficacy of the commercially available probiotic mixture VSL#3 on patients with mild to moderate ulcerative colitis. VSL#3 contains eight probiotic lactic acid bacteria in high numbers, including four members of the Lactobacillus genus, three species of Bifidobacterium and *Streptococcus thermophilus*. Treatment with this mixed species supplement resulted in disease remission in significantly more patients compared to conventionally treated patients[44] or the placebo group,[45] although the basis for this efficacy remains unknown. Based on this collective data it is tempting to speculate that at least some of the bacterial species promoted in parallel with LGG in our TIPS study exert a concerted impact on asthma development, potentially through a similar mechanism, i.e., the appropriate activation of $CD4^+Foxp3^+$ regulatory T cells. Although provocative, further functional analysis of these microbiota is necessary to determine the fundamental mechanism by which probiotic supplementation benefits the human host.

In summary, our work and the recently published reports presented here suggest that the structure of the microbiota can be manipulated through supplementation with probiotic species to promote communities that are more resilient and putatively beneficially modulate host immune responses. Based on the collective data to date, in the case of the TIPS study we propose the following scenario: the daily supplementation with LGG beginning very shortly after birth results in a high abundance of this species in the infant intestine. High numbers of LGG shapes the gastrointestinal niche, for example, by altering the pH of the environment through lactic acid production, thus facilitating colonization by a diversity of beneficial bacterial species who are fittest in this specific environment. Consequently, a relatively diverse, even and functionally redundant community structure evolves that educates the developing immune response and is protective against sub-population overgrowth and gastrointestinal infection. Putatively, species within this microbiota maintain immune homeostasis, through the generation and promotion of anti-inflammatory regulatory T cells. The next step in these studies is to define the exact microbial and host mechanism(s) by which supplementation elicits a human host beneficial effect, whether this intervention ultimately results in improved allergic disease development outcomes in all supplemented infants, and to identify the factors characteristic of those GI microbiota refractory to microbial manipulation.

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